

Serum Zinc, Calcium and Albumin Levels in Pulmonary Tuberculosis Patients Co-Infected with HIV

¹M. Muthuraj, ²S. Kamatchiyammal, ³B. Usharani, ⁴S. Manupriya,
⁵A.R.Niranjana Ayyappan and ⁵K. Divyalakshmi

^{1,4}Department of Microbiology, Government Hospital for Chest Diseases, Puducherry, India

²Departments of Microbiology, NEERI, CSIR Campus, Tharamani, Chennai, Tamilnadu, India

³Departments of Microbiology, Vector Control Research Centre, Puducherry, India

⁵J.J. College of Arts and Science, Pudukottai, Tamil Nadu, India

Abstract: Zinc is a component of both structural and catalytic proteins of HIV. Zinc deficiency affects host defense by decreasing phagocytosis and reducing the number of circulating T cells. The present study was carried out to estimate serum zinc, calcium and albumin levels in newly detected adult active pulmonary tuberculosis patients co-infected with HIV and to compare them with the levels in controls (healthy family members). Standard methods were adopted to collect an early morning fasting blood sample for zinc and calcium (by Inductively Coupled Plasma-Optical Emission Spectrophotometer) and albumin (estimated by the bromocresol green method). Partec CyFlow Counter analysis of CD4 counts for Normal, Pulmonary Tuberculosis patient, PTB patient co-infected with HIV and HIV patient are 1773 / ul, 793 / ul, 379/ ul and 550 / ul respectively. Serum albumin levels of PTB patient co-infected with HIV (1.8 g/dL) had lower level than normal (3.78 g/dL) healthy individual. The zinc and calcium levels of PTB patient co-infected with HIV (Zn: 467.5bp, Ca: 20750bp) lower than the zinc and calcium levels (Zn: 1251bp, Ca: 41580bp) of normal (control) healthy individual. All three parameters were significantly low in active PTB patients with HIV. These changes may be attributable to nutritional factors, enteropathy and acute phase reactant proteins. Hence, the National AIDS Control Organization (NACO) in India is providing nutritional supplements to those HIV-infected cases inducted for antiretroviral therapy and nutritional counseling for others as a part of a national policy.

Key words: Pumonary Tuberculosis • Mycobacterium tuberculosis • HIV • Zinc • Calcium • Albumin

INTRODUCTION

Tuberculosis (TB) is a global public health problem, responsible for more than 2 million deaths each year [1] and the majority of these individuals live in developed countries where Human Immunodeficiency Virus (HIV) infection is spreading rapidly. Every year 1.8 million people in India develop tuberculosis (TB). India accounts for one-fifth of the global TB incidences are estimated to have the highest number of active TB cases the countries of the world. TB kills more adults than any other disease, accounting for almost 400,000 deaths annually. It mainly afflicts are in the economically years of their lives (15-54 years), huge social and economic disruption [2]. The association between TB and malnutrition is well recognised; TB can lead to malnutrition and

malnutrition may predispose to TB[3]. Weight loss is one of the fundamental signs both in HIV-positive and HIV-negative tuberculosis (TB) patients [4]. Malnutrition and wasting are associated with TB and HIV infection. Coinfection with one may potentially exacerbate the wasting that occurs in the other [5]. Micronutrient deficiency, such as zinc deficiency, leads to impaired immunity and thereby increases susceptibility to infections such as TB [6].

Zinc is a component of both structural and catalytic proteins of HIV. Zinc is required for the activity of reverse transcriptase and the production of infectious virus [7] and may inhibit HIV replication through binding to the catalytic site of HIV protease [8]. Zinc deficiency affects host defense by decreasing phagocytosis and reducing the number of circulating T cells [9]. Also, zinc deficiency

has been observed in HIV infection at various stages of the disease [10] and may be a cofactor for the progression of the disease [11]. Calcium levels exhibited its aggravating effect by either interfering with zinc absorption from the gastrointestinal tract, or by interacting with zinc at specific cellular sites within the body [12,13]. Thus, coinfection with HIV and TB results in a number of micronutrient deficiencies that may increase vulnerability to immune dysfunction [14].

Because of the limited data on the serum level of zinc and calcium in patients with adult pulmonary tuberculosis (PTB) with and without HIV infection, it was decided to study the serum zinc, calcium and albumin levels in such patients and compare them with the levels in healthy controls. Thus, the effective management of diseases, including TB, therefore requires detailed evaluation of the nutritional status since this can help prevent or modify many complications of diseases and also help in making projection of the interaction of nutritional status on the clinical course of the disease.

MATERIALS AND METHODS

Patients and Selection: The blood and sputum sample was collected from the Tuberculosis(TB) patient, Tuberculosis(TB) patient co-infected with HIV and HIV patient admitted in Government Hospital for Chest Disease at Puducherry and the samples was processed in Department of Microbiology, State TB Training and Demonstration Centre (Intermediate Reference Laboratory) of this hospital for RT PCR, Auto analyzer, Isolation and identification, DNA extraction, PCR amplification, Inductive Coupled Plasma (ICP) analysis (Atomic absorption spectrophotometer) and agarose gel electrophoresis.

Blood collection and CD4 counting: After obtaining informed consent, two aliquots of blood were collected by sterile venipuncture into tubes containing EDTA anticoagulant. CD4+ T-cell enumeration was performed using the Partec CyFlow Counter. The CyFlow Counter uses a 'no lyse, no wash' procedure for CD4 counting [15]. Fifty microlitres of EDTA-anticoagulated blood were added to 10 µl of monoclonal antibodies. After 15 min of incubation, 1ml of no lyse dilution buffer was added and the sample tube was attached to the Partec CyFlow Counter for automated counting. Results were available in two minutes and were expressed in a histogram (CD4+cells/ µl).

RNA extraction: Take approximately 1ml of blood in a clean tube containing 5ml of erythrocyte lyses (EL) buffer, mixed well and incubated in ice condition for 10 minutes. Centrifuged the sample at 600rpm for 10 minutes at 4°C and discarded the supernatant. Centrifuge at 1000 rpm after addition of 2ml of EL buffer and discarded the supernatant. Add 300µl of RLT buffer to the pellet and it was transferred to the Q1A shredder mini spin column, centrifuged for 2 minutes. Add 350µl of 70% ethanol to that mixture and it was transfer to another fresh Q1A spin column. Centrifuged the sample for 15 seconds, discarded the supernatant and add 500 µl of RPE buffer, again it was centrifuged for 30 minutes and the pellet was transferred to the fresh Q1A shredder spin column. Add 40µl of RNase to the pellet, centrifuged for 1 minute and discarded the supernatant. The pellet containing the RNA was used as template for RT PCR analysis.

RT-PCR Analysis: The volumes of super mix and internal control per reaction multiply with the number of sample which includes the number of controls, standards and sample prepared. Molecular grade water is used as the negative control. For reasons of imprecise pipetting, always add an extra virtual sample. Mix completely then spin down briefly in a centrifuge. Pipette 3ul of mater mix with micropipette of sterile filter tips to each of the RT-PCR reaction plate / tube. Separately add 5ul RNA sample supernator positive and negative controls to different reaction plate / tubes. Immediately close the plate / tubes to avoid contamination. Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes. Set the reaction in the eppendorf instrument in 45°C for 10 minutes for 1 cycle,95°C for 15 minutes for 1 cycle, 95°C for 15 seconds, 60°C for 60 seconds for 40 cycles and Fluorescence was measured at 60°C.

Mycobacterium DNA Extraction: One loopful of culture was taken in 100µl of sterile distilled water and was homogenized. The entire homogenized samples were treated with 50 µl of lysozyme (10mg/ml) at 37 °C for overnight incubation. 70µl of 14% SDS and 6µl of Proteinase K (10 mg / ml) was added to precipitate the proteins and was incubated at 65°C for 15 minutes. 10 µl of 5M NaCl and 80µl of CTAB/NaCl were added to remove the polysaccharides and unwanted residues and was incubated at 65 °C for 10 minutes. 800 µl of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added to

remove the proteins from preparation of nucleic acid. The chloroform denatures the proteins while Isoamylalcohol reduces foaming during extraction and facilitates the separation of the aqueous and organic phase. Centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and 600µl of Isopropanol was added to precipitate the DNA and incubated overnight at -20° C. Centrifuged at 12000 rpm in 4 °C for 10 minutes. The pellet was washed with 70% ethanol to remove any remaining solutes. The pellet was air-dried and was dissolved in 20µl of 1x TE buffer [16].

PCR Amplification: The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf Gradient Cycler). This confirms the template DNA as *Mycobacterium tuberculosis*. The PCR reaction was set up as follows using the primer for *Mycobacterium IS6110* amplification F 5'GTGAGGGCATCGAGGTGG 3' (10pmol/µl) R 5'CGTAGGGCGTCGGTCA CAAA 3' (10pmol/µl). The PCR cycling parameters were 94°C for 5 minutes; followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 72 °C for 1 minute; and a final extension of 72°C for 10 minutes. The PCR was then kept at hold at 4°C for 15 minutes. The amplified PCR product was withdrawn from thermal cycler and run on a 2% Agarose gel in TAE buffer. The Ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc.

Agarose Gel Electrophoresis: The gel running tray was placed in a clean gel casting tray to form the gel uniformly and the comb was fixed at one end. 400mg of agarose (2%) powder was added to 20ml of 0.75x TAE and was boiled for few seconds to dissolve the agarose completely. Less than 1µl of Ethidium bromide (0.5mg/ml) was added into the hand bearable heat 250 ml conical flask containing melted agarose gel and was poured into the gel running tray. 1µl of gel loading dye was transferred into a 5x5 cm size Para film. To it 5µl of polymerized DNA was added and was mixed thoroughly. The whole volume aliquot of amplified sample with gel loading dye was loaded into a well of 2% agarose gel in 0.75x TAE buffer and was subjected to electrophoresis for 30 minutes at 100 volts. The gel was observed under UV Transilluminator for specific DNA bands and was photographed. The DNA bands were identified according to the size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane.

Protein Digestion for ICP Analysis: Pretreated the serum samples with nitric acid before treating it with perchloric acid and avoid the repeated fuming with perchloric acid. Never let the samples to evaporate to dryness. Take approximately 5-10ml of pretreated serum sample in a clean beaker (If sample is not acidified, treat it with nitric acid and evaporate on hot plate to 15ml to 20ml.). Add 10ml each of concentrated nitric acid and perchloric acid in a cooling condition. Evaporate gently on a hot plate until dense white fumes of perchloric acid appear. If necessary, add 10ml concentrated nitric acid to complete digestion. Let the beaker is allowed to cool and dilute to about 50ml with water (Boil to expel any chlorine or oxides of nitrogen). Filter the solution through whatmann paper and the filtrate can be used for the Inductive coupled Plasma (ICP) analysis.

Serum Zinc and Calcium Analysis: The acid digested samples were washed several times with milli Q water and the samples were diluted to 50ml. Take 50 µl of yttrium standard with 0.1ml of nitric acid in a separate clean conical centrifuge tube and make up to 50 ml using sterile milli Q water. Approximately 1.5ml of the sample/min was introduced into the (Perkin Elmer analyzer) Inductively Coupled Plasma-Optical Emission Spectrophotometer. The emission was measured at its own specified wave length.

Serum Albumin Analysis: Take approximately 10 µl of serum sample of each patient to a clean sterile test tube and add 1.0ml of BCG reagent to this tube. Mix gently and allow reacting at room temperature for ten minutes. Measure the absorbance of test and standard after ten minutes against reagent blank at 630nm (600-650nm). The amount of serum albumin is calculated by

$$\text{Albumin concentration (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 5$$

RESULTS

The CD4 count levels of Tuberculosis (TB) Patient co-infected with chronic HIV had lower CD4 counts than those who had TB, HIV alone. Serum of normal patients had considerably higher CD4 count than others. Fig.1 shows the CD4 counts of Normal (1773 / ul), Tuberculosis (793 / ul) patient, TB patient co-infected with HIV (379/ ul) and HIV (550 / ul) patient. RT PCR analysis (Fig.2, 3:) confirms the HIV status of the blood sample.

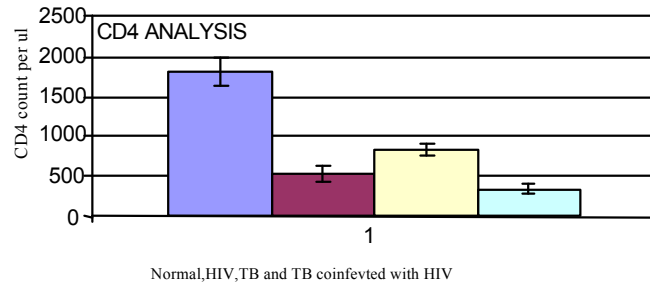


Fig. 1: CD 4 count analysis using Partec CyFlow Counter

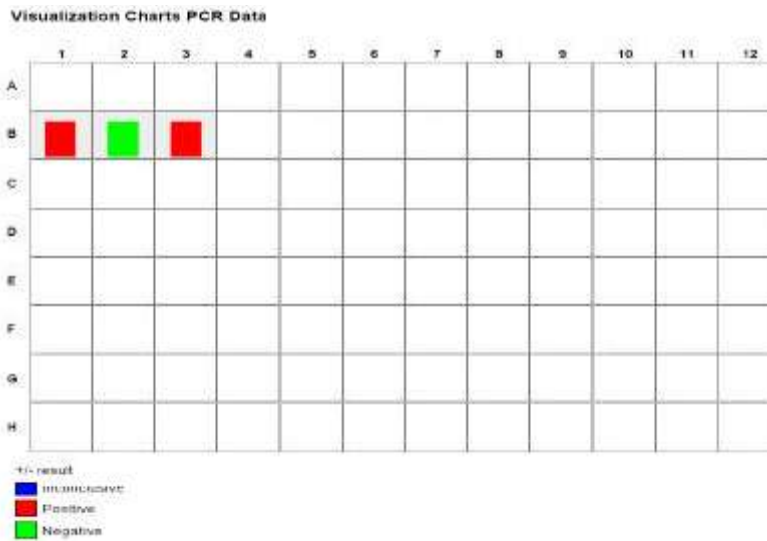


Fig. 2: RT-PCR – Visualization chart

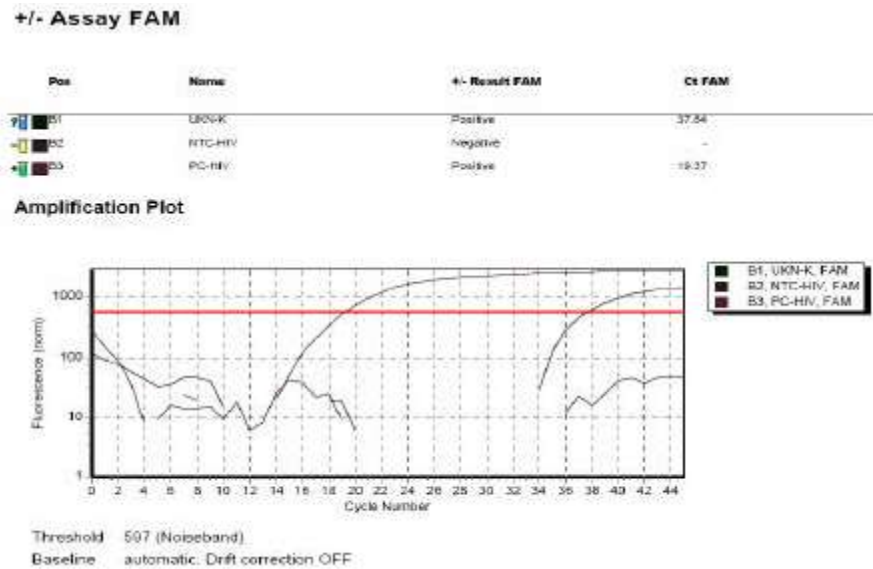


Fig. 3: RT-PCR Amplification plot

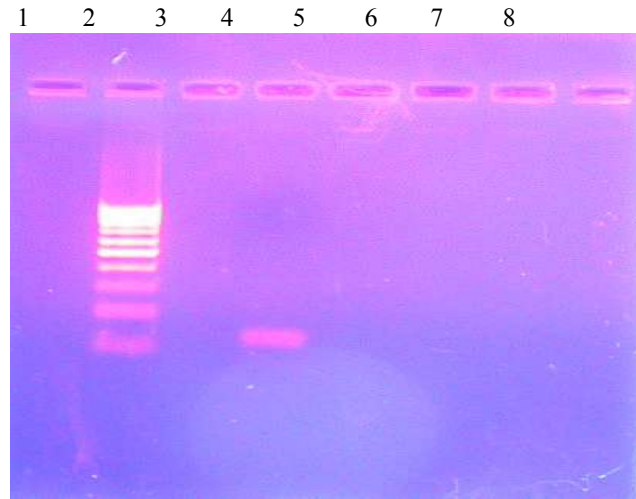


Fig.4: Lane 4:123 bp products amplified with IS6110 primer.
Lane 2: 100 bp ladder

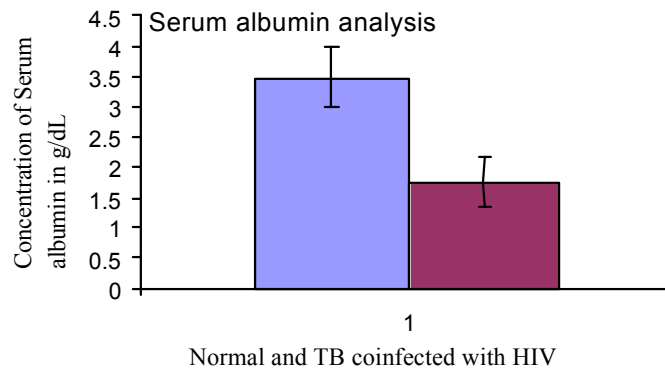


Fig.5: Serum albumin levels in normal and PTB co infected with HIV patients

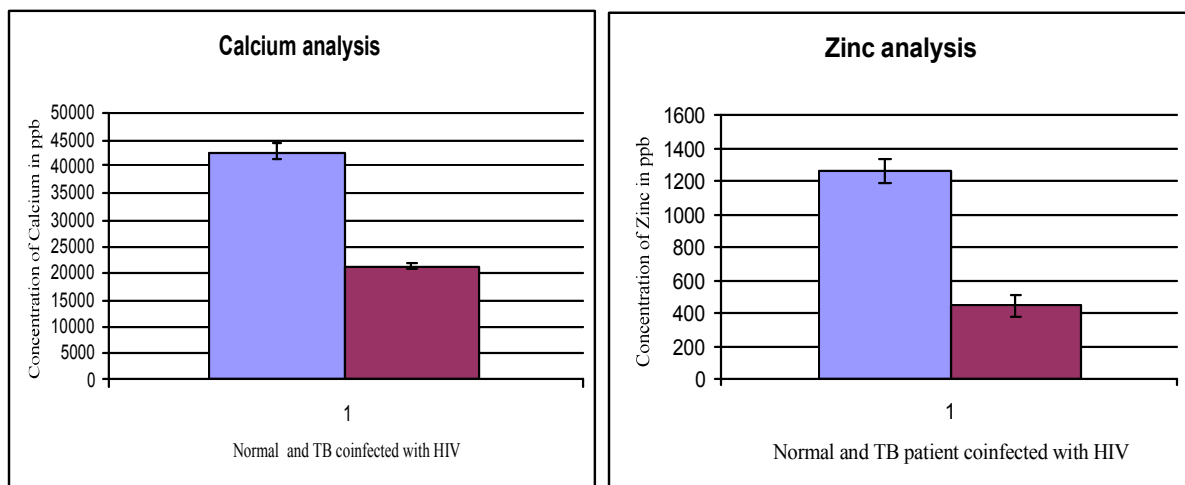


Fig.6: Serum Zinc and Calcium Analysis using Inductively coupled plasma analysis (Optical emission Spectrometer)

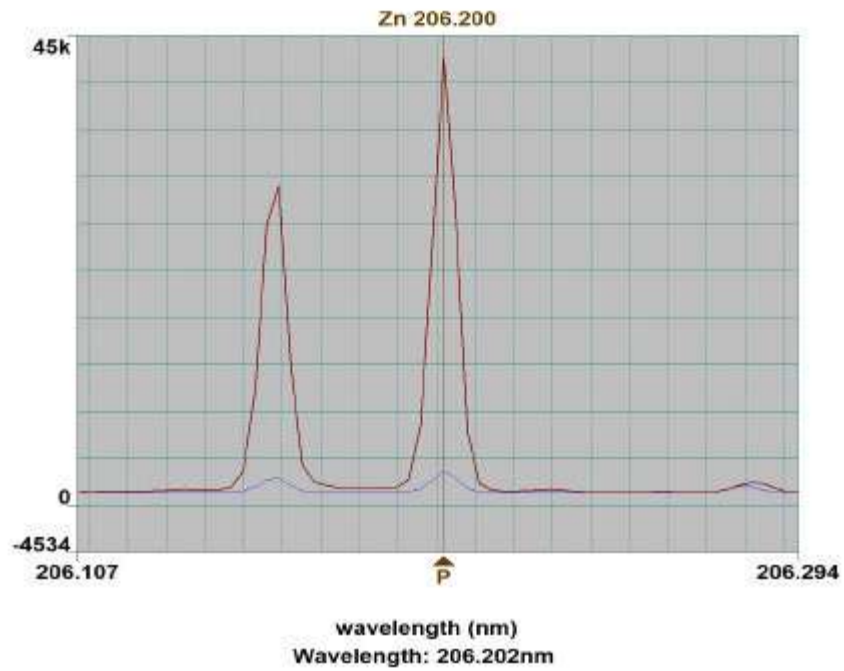


Fig .7: Zinc Analysis of Inductively coupled plasma analysis (Optical emission Spectrometer). in Normal healthy individual

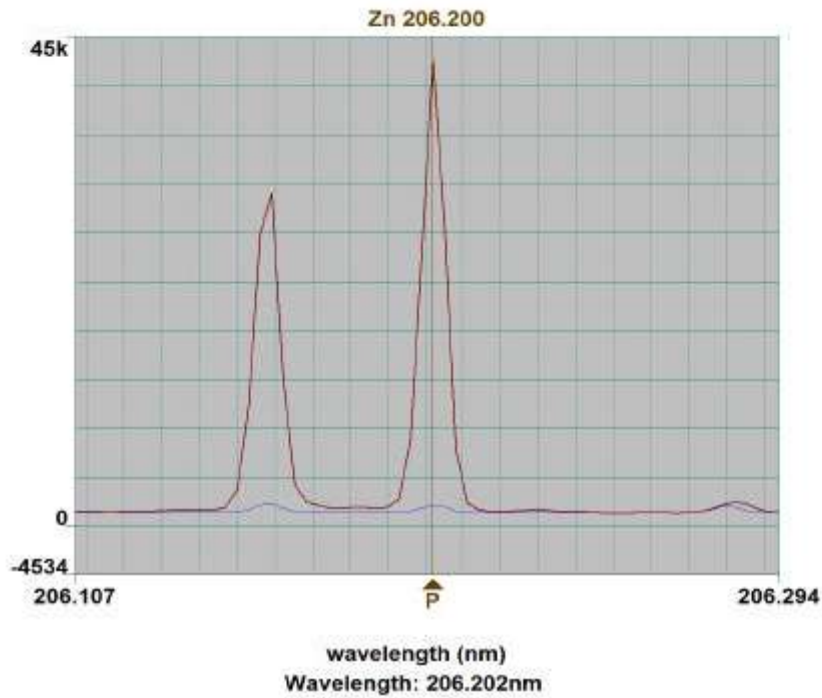


Fig .8: Zinc Analysis of Inductively coupled plasma analysis (Optical emission Spectrometer). in Pulmonary Tuberculosis co infected with HIV.

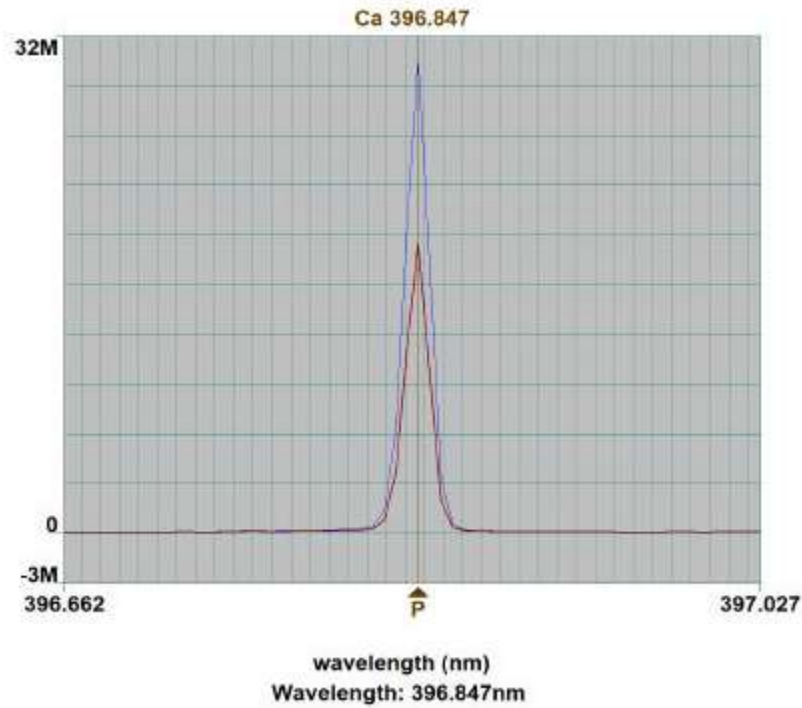


Fig. 9: Calcium Analysis of Inductively coupled plasma analysis (Optical emission Spectrometer) in Normal healthy individual

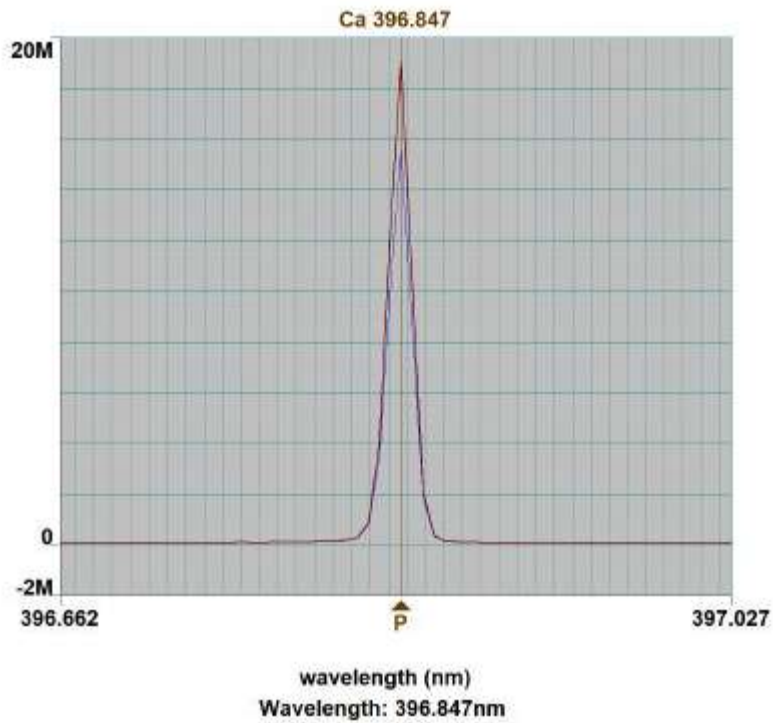


Fig.10: Zinc Analysis of Inductively coupled plasma analysis (Optical emission Spectrometer). in Pulmonary Tuberculosis co infected with HIV

Mycobacterial DNA was isolated from the L.J. medium slant and was subjected to PCR amplification using species specific primers, targeting the insertion sequence IS6110 (Mtb 5'G TGAGGGCATCGA GGTGG 3') (Mtb 5'CGTAGGCGTCCGGTCACAAA 3') for conforming the *M.tuberculosis*. The PCR product was run on a 2% agarose gel. A clear band was formed at 123bp region confirming the presence of *M.tuberculosis* in the sputum specimen (Fig. 4). Serum albumin levels (Fig. 5) of TB patient co-infected with HIV (1.8 g/dL) had lower level than Normal (3.78 g/dL). The zinc and calcium levels of PTB patient co-infected with HIV is (Zn: 467.5bp, Ca: 20750bp) lower than the zinc and calcium levels (Zn: 1251bp, Ca: 41580bp) of normal (control) healthy individual. Fig. 6 shows the Serum zinc and calcium levels of Normal (control) and PTB patient co-infected with HIV. Fig.7-10 represents the chromatogram of the zinc and calcium analysis of Normal (control) and PTB patient co-infected HIV using Inductively coupled plasma analysis (Optical emission Spectrometer).

DISCUSSION

This investigation has revealed that the pulmonary TB co-infected with HIV (AIDS) subjects with CD4 T lymphocyte count less than pulmonary tuberculosis of the patient was checked with AFB smear and PCR amplification of insertion sequence using IS6110 primer for screening of tuberculosis. In the present study, serum zinc, calcium and albumin zinc levels were significantly reduced in patients with PTB irrespective of their HIV status compared to healthy controls. The possible causes for the low serum zinc and albumin in PTB patients were considered to be nutritional factors, enteropathy and acute phase reactant proteins [17]. The hepatic synthesis of acute phase reactant proteins is induced by cytokines such as interleukin-6 and tumor necrosis factor [18], which inhibit the production of serum albumin and cause dramatic shifts in the plasma concentration of certain essential micro nutrients and albumin. The level of serum zinc observed among TB patients was significantly lower than that of control. Also, this was likely due to the redistribution of zinc from plasma to other tissues, or a reduction of the hepatic production of the zinc-carrier protein macroglobulin and to a rise in the production of metallothionin a protein that transports zinc to the liver [19].

Since HIV-infected adults with PTB had significantly low zinc, albumin and BMI, this subgroup may potentially benefit from nutritional interventions. Several studies

have revealed that micronutrient supplementation to patients with active TB and HIV improves their health by increasing CD4+ count [20], increasing their weight and improving the efficacy of their drug treatment [21] in addition to decreasing opportunistic infections. Interestingly, intake of zinc beyond a certain level was associated with increased relative risk for disease progression [22], although another previous study failed to replicate this finding [23].

To conclude, the nutritional status of patients with active PTB is poor when compared with healthy controls. Reductions in the concentration of plasma zinc and albumin as well as wasting were significantly greater in PTB patients with HIV infection than in non-HIV PTB patients. The study thus indicates the need for therapeutic supplementation of zinc and proteins to patients with active TB irrespective of HIV status. Currently the National AIDS Control Organization (NACO) in India is providing nutritional supplements to those enrolled in the ART Program, while counseling on nutrition is provided for other HIV-infected cases as part of a national policy.

REFERENCE

1. WHO, 2003. Treatment of tuberculosis. Guidelines for National Programmes. 3rd ed. Geneva: World Health Organization.
2. Chambers, H.F., D. Moreau and D. Yajko, 1995. Can *penicillins* and other beta-lactam antibiotics are used to treat tuberculosis. *Antimicrob Agents Chemother*, 39: 2620-4624.
3. Macallan D., 1999. Malnutrition in tuberculosis. *Diagnostic Microbiology and Infectious Diseases*, 34: 153-157.
4. Van Lettow, M., A.D. Harries and J. Kumwenda, 2004. Micro nutrient malnutrition and wasting in adults with pulmonary tuberculosis with and without HIV co-infection in Malawi. *BMC Infect. Dis.*, 4: 2334-2344.
5. Swaminathan, S., C. Padmapriyadarsini and B. Sugumar, 2008. Nutritional status of persons with HIV infection, persons with HIV infection and tuberculosis and HIV negative individuals from southern India. *Clin. Infect. Dis.*, 46: 946-948.
6. Karyadi, E., W. Schultink and R.H.H. Nelwan, 2000. Poor micro nutrient status of active pulmonary tuberculosis patients in Indonesia. *J. Nutr.*, 130: 2953-2958.

7. Tanchou, V. *et al.* 1998. Role of the N-terminal zinc finger of human immunodeficiency virus type 1 nucleocapsid protein in virus structure and replication. *Journal of Virology*, 72: 4442-4447.
8. Zhang, Z.Y. *et al.* 1991. Zinc inhibition of rennin and the protease from human immunodeficiency virus type 1. *Biochemistry*, 30: 8717-8721.
9. McMurray, D.N., R.A. Bartow, C.L. Mintzer, *et al.* 1990. Micronutrient status and immune function in tuberculosis. *Ann. N.Y. Acad. Sci.*, 587: 59-69.
10. Febris, N., E. Mocchegiani, M. Galli, *et al.* 1988. AIDS, zinc deficiency and thymic hormone failure. *JAMA*, 259: 839-840.
11. Falutz, J., C. Tsoukas and P. Gold, 1988. Zinc as a cofactor in human immunodeficiency virus induced immunosuppression. *JAMA*, 259: 2850-2851.
12. Forbes, R.M., 1960. Nutritional interactions of zinc and calcium. *Federation Proc.*, 19: 643.
13. Hoekstra, W.G., 1964. Recent observations on mineral interrelationships. *Federation Proc.*, 23: 1068.
14. Taneja, D.P., 1990. Observation on serum zinc in patients of pulmonary tuberculosis. *J. Indian Med. Assoc.*, 88, 280-281.
15. Greve, B., U. Cassens, C. Westerberg, W. Ghde, W. Sibrowski and D. Reichelt, 2003. A new non-lyse, no wash flow-cytometric method for the determination of CD4 T cells in blood samples. *Transfus. Med. Hemother*, 30: 8-13.
16. Mani, C., N. Selvakumar, V. Kumar, S. Narayanan and P.R. Narayanan, 2003. Comparison of DNA sequencing, PCR-SSCP and PhaB assays with indirect sensitivity testing for detection of rifampicin resistance in *Mycobacterium tuberculosis*. *International Journal of Tuberculosis and Lung Diseases*, 7: 625-659.
17. Karyadi, E., C.E. West, W. Schultink, *et al.* 2000. A double-blind placebo-controlled study of vitamin A and zinc supplementation in persons with tuberculosis in Indonesia: effects on clinical response and nutritional status. *Am. J. Clin. Nutr.*, 75: 720-727.
18. Xing, Z., J. Gauldie, G. Cox, *et al.* 1998. IL-6 Is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J. Clin. Invest.*, 101: 311-320.
19. Gabay, C. and I. Kushner, 1999. Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.*, 340: 448-454.
20. Jon, D.K., M. Adriana, P. Joseph, *et al.* 2006. Micronutrient supplementation increases CD4 count in HIV-infected individuals on highly active antiretroviral therapy: a prospective, double-blinded, placebo controlled trial. *J. Acquir. Immune Defic. Syndr.*, 42: 523-528.
21. Range, N., 2006. The effect of multivitamin/mineral supplementation on mortality during treatment of pulmonary tuberculosis: a randomized two-by-two factorial trial in Mwanza, Tanzania. *Br. J. Nutr.*, 95: 762-770.
22. Baum, M.K., G.S. Posner and A. Campa, 2000. Zinc status in human immunodeficiency virus infection. *J. Nutr.*, 130(5S): 1421S-1423S.
23. Neera, S., 2002. A clinical review of micronutrients in HIV infection. *J. Int. Assoc. Phys. AIDS Care*, 1: 63-75.